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Structure of glycogen

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STRUCTURE OF GLYCOGEN

by

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INTRODUCTION AND REVIEW

The study of native glycogen is a little over ten years old. This contention may seem absurd in the light of the first report on "matiere glycogene animale," which appeared over a century ago; yet improved methodology has allowed for a modern era of investigation into the properties of glycogen. Glycogen's first century, the things done to it and in its name, is well reviewed by Manners (1). More recent aspects of glycogen structure, isolation, and enzymology are the subject of two symposia (2, 3).

Manners (1) stated that at the time of his review in 1957 the most commonly used isolation procedure was tissue digestion in hot, concentrated alkali followed by several alcohol precipitation steps. Other often used methods involved tissue disruption in trichloroacetic acid solution or hot water. In 1958, Orrell and Bueding (4) introduced a cold-water isolation procedure similar to that initially reported by Lazarow (5). Other cold-water procedures have been reported by Drochmans (6) and by Mordoh *et al.* (7). Bueding and Orrell describe their cold-water procedure more fully in a later publication (8). Employing the analytical ultracentrifuge, they demonstrated in a companion paper (9) that glycogen isolated from rabbit liver by their procedure is much larger (with sedimentation coefficients ranging over 1500) than glycogens isolated by methods utilizing heat

and/or extremes of pH. Drochmans (6, 10) has demonstrated by microscopy and turbidity measurements the degradation of cold-water isolated glycogens under acidic conditions mimicking those of the trichloroacetic acid isolation scheme. The glycogen he initially isolated was composed of large, rosette-shaped particles he termed "α-particles." Stadhouders (11) demonstrated that glycogen isolated by cold-water extraction is morphologically indistinguishable from that observed in tissue thin-section. In addition to size differences, Childress et al. (12) contend that cold-water isolated glycogen behaves differently from alkali-isolated glycogen in kinetic measurements utilizing phosphorylase a. The cold-water isolation procedures, yielding glycogen which is, at most, little degraded from its physiologically active condition, and the application of analytical ultracentrifugation and electron microscopy to the isolated material are the leading techniques that in the past several years have allowed advances in the understanding of glycogen.

A recent article by French (13) discusses some of the factors involved in glycogen structure and the limitations on current understanding of glycogen structure. One of the limiting factors is the lack of knowledge about the distribution of structural features within the molecules comprising the sample under study, a factor which is important in light of the Meyer model (14) whose structure is random within the limitations of the specificities of glycogen-metabolizing

enzymes and steric factors. A structural feature of particular interest is the branching density, the closeness of branching bonds within glycogen.

The facts that the average chain length in most glycogens is about 12 glucose units and the internal chain length is about 3 glucose units have been long known and are incorporated in the Meyer model. The average chain length values give no indication of the variation in chain lengths. An early indication of chain lengths considerably longer than 12 was the observance of iodine complexes with some glycogen samples having an absorption maximum at a higher wavelength than is usually observed. The wavelength of the iodine-complex absorption maximum increases with increasing chain length (15). Considering the other end of the distribution, degradation of glycogen β-limit dextrin with Bacillus subtilis amylase did not yield any products detectable by paper chromatography (16). Considering the product specificity of the B. subtilis amylase, i.e., preferential production of maltohexaose and maltoheptaose (16) and the formation of 6^2-α-maltosylmaltotriose as the smallest singly-branched α-dextrin (17), it would seem that the occurrence of internal chain lengths of 8 or more is rare. Another paper contributing to a description of the internal chain length distribution in glycogen describes the finding of multiply-branched oligosaccharides, containing 2, 3, and perhaps more branch points, resulting from the degradation of glycogen by salivary

α-amylase (18). The structures indicated by Roberts and Whelan (18) have internal chain lengths of 1 and 2 glucose units. Heller and Schramm (19) reported the finding of large, multiply-branched "macrodextrins." These authors were not the first to observe an amylase-resistant polyglucose residue after extensive treatment of glycogen with α-amylase (20), but they did indicate that the macrodextrin is an intrinsic part of glycogen rather than a distinct polyglucose. Heller and Schramm were seeking to repeat the work of Roberts and Whelan (18) but employing gel filtration designed to detect possible high molecular weight limit dextrins. Widely differing amounts of high molecular weight dextrins, both in total and in the various fractions ranging from the lowest, D.P. 7-50, to the highest, D.P. >100, were obtained after similar enzymatic treatments of different glycogens and amylopectin. Heller and Schramm conclude that the amount of macrodextrin and its size are a measure of the distribution pattern of branch points in glycogen.

The observation of macrodextrin formation raised the questions: Where in the glycogen molecule do macrodextrins occur and what is their origin?

The question of where in the glycogen molecule the macrodextrins occur has been approached by observing ultracentrifugally the size decrease of the parent molecule during α-amylolysis. The macrodextrin is a large, "limit" dextrin. Limit is operationally regarded as some extent of reaction

after which further reaction occurs at a much slower rate. For example, β-limit dextrin does not mean that further treatment with β-amylase will never yield any maltose, but it does mean that the rate of further maltose liberation is much less than the initial rate. Generally, limit means that further reaction proceeds at a rate considerably less than one percent of the initial rate.

β-amylase, a strictly exo-enzyme, removes maltose units from the chemical periphery of glycogen down to the first encountered branch point, leaving a limit dextrin containing all the original branch points and about one half the original number of glucose units. The branching density could be thought to increase from that density around the branches furthest removed from the reducing group to the greatest density near the reducing group. This gradation could arise if the specificity of the branching enzyme allows for placement of a branch as close as the second glucose unit away from an existing branch and if greater distance between branch points (lesser branching density) is demanded by the increasing density of glucose units as the polymer grows and is continually branched. A less regimented synthetic scheme could allow for random placement within the molecule. Such randomly-placed macrodextrin regions would be separated by less densely branched regions which would be more easily hydrolyzed. According to the scheme of increasing branching density toward the reducing end of the molecule, the size of

the parent molecule would decrease slowly during α-amylolysis with macrodextrins being a late product of the reaction. Alternatively, the size of the parent molecule containing randomly-placed macrodextrins would decrease more rapidly as the more easily hydrolyzed inter-macrodextrin regions are cleaved, with the production of macrodextrin early in the reaction. The two alternative concepts can be tested by observing the size decrease of the parent glycogen molecule and the release of macrodextrins during enzymatic degradation.

Glycogen, a natural polymer, is a population of similarly constituted molecules that are identical in monomer unit and type of bonding but that differ in such factors as branching pattern, shape, and molecular weight. As early as 1942, Bridgman (21) reported maximum ordinate sedimentation coefficients and sedimentation coefficient distributions for rabbit liver glycogens prepared by either trichloroacetic acid or alkali methods. Distributions of sedimentation coefficients have since been reported for glycogens from widely different sources (12, 22, 23, 24, 25). Such distributions of cold-water isolated glycogens are either positively skewed or bimodal. Any glycogen preparation exhibits a distribution of sedimentation coefficients due to differences in shape and molecular weight; nonetheless, a single number was desired for convenience as a size measure of undegraded glycogen and glycogen in various states of enzymatic degra-

dation. The maximum ordinate sedimentation coefficient was taken as an indicator of the central tendency of the distribution under investigation, and comparisons between such coefficients for different populations were taken as an average of the same comparison among the members of those populations. The decrease in the maximum ordinate sedimentation coefficient has been taken as an index of the decrease in molecular size.

Attempts have been made to translate sedimentation coefficient distributions into molecular weight distributions. Indeed, Bridgman (21) translated his sedimentation coefficient distribution using three different molecular weight scales, each based upon a different assumption as to diffusion coefficient or shape. It might be well to heed the suggestion made by Meyer (26) that for many homologous polymeric mixtures knowledge of the order of molecular weight is sufficient and that in many cases molecular weights may be completely dispensed with and characterization made by direct use of experimental parameters which depend wholly or in large part upon molecular weight. No attempt at obtaining a molecular weight distribution has been made. A graphic method for estimating the magnitude of molecular weights for any examined glycogen preparation from the maximum ordinate sedimentation coefficient of that preparation will be presented in a later section.

Some modest beginnings have been made in seeking the

origin of the macrodextrins. Four enzymes account for the bulk of intracellular glycogen metabolism: glycogen synthetase (EC 2.4.1.11 UDPglucose:α-1,4-glucan α-4-glucosyltransferase), branching enzyme (EC 2.4.1.18 α-1,4-glucan:α-1,4-glucan 6-glycosyltransferase), phosphorylase (EC 2.4.1.1 α-1,4-glucan:orthophosphate glucosyltransferase), and debranching activity (EC 3.2.1.33/EC 2.4.1.25 amylo-1,6-glucosidase/ oligo-1,4→1,4-glucantransferase) (3). The synthetase and branching enzymes sythesize glycogen, whereas phosphorylase and debranching enzymes are responsible for its degradation; this is a simplified picture. Alternative enzymes and directions of reactions are possible; e.g., see discussion in CIBA symposium on glycogen (27) and a recent article by Huijing et al. (28). Densely-branched macrodextrin regions may be accounted for on the basis of statistical fluctuation in the placement of a given branch point in relation to already existing branch points during glycogen synthesis. In such a circumstance, the amount of total branching and, hence, of macrodextrin would depend upon the relative activities of branching and elongating enzymes. Smith (29) reports the dependence of the amount of branching upon the relative amounts of branching and elongating enzymes for an in vitro system. He reports nothing concerning macrodextrins or any other branched regions in his synthetic glycogen.

There seem to be no reports concerning the control of

branching enzyme. If there is no way of shutting off branching activity or of compartmentalizing it from the dynamic glycogen pool, then glycogen in whatever state of synthesis or degradation and regardless of net glycogenesis or glycogenolysis would be continuously exposed to branching enzyme. Thus even though a less densely branched glycogen might be synthesized during a period of relatively high synthetase activity, continued exposure of the glycogen to branching enzyme after the synthetase activity has decreased could be expected to yield a glycogen with an equilibrium branching pattern and density. But the branching enzyme is not the only enzyme affecting 1→6 bonds. There seem also to be no reports on the control of debranching activity. If this enzyme, too, has somewhat free rein on glycogen, then the branching pattern and density of all but newly synthesized glycogen will reflect the activities and specificities of branching and debranching enzymes. Huijing et al. (28) have proposed that what branching does occur in type IV glycogenosis (amylopectinosis) is accomplished by the reverse action of debranching enzyme. As the name suggests, the glycogen stores in type IV glycogenosis are much less densely branched than normal glycogen, i.e., more nearly resembling amylopectin. Amylopectin yields no macrodextrin (19). If the complexity of branching formed by the debranching enzyme acting in the reverse of its usually considered reaction is an indication of the most complex branching arrangement it

can degrade, then the combined action of branching and debranching enzymes would produce a glycogen whose branching complexity would increase with time. The picture would be only slightly modified if branching and debranching enzymes are controlled in some fashion analogous to the control of synthetase and phosphorylase. If, as suggested above, branching enzyme can produce a complexity of branching which debranching enzyme cannot degrade, then cyclic rather than concerted action of branching and debranching enzymes would still result in an increase in branching complexity with time. Addressing, then, the question of macrodextrin origin, the glycogen stores of a population of protozoans were cycled by successive feeding and fasting, and the change in branching complexity was measured.

MATERIALS AND METHODS

Reagents

Shellfish glycogen (Lot #75B-1600) was obtained from Sigma Chemical Co. Sephadex was a product of Pharmacia Fine Chemicals, Inc.; proteose peptone and yeast extract were products of Difco Laboratories. Porcine pancreatic α-amylase and sweet potato β-amylase were obtained from Worthington Biochemical Corp. Other chemicals were of reagent grade.

Organism and Culture

Organism

The protozoan Tetrahymena pyriformis [A. M. Elliot strain $WH_{14}(WH_{II})$, syngen 1, mating type II] was obtained from the culture maintained at the microscopy laboratory, Department of Biochemistry and Biophysics, Iowa State University.

Culture conditions

The culture was initially maintained and grown on medium A, Table 1. A simpler medium, medium B, Table 1, was employed for maintenance and growth at the later time when the "cycling" experiments were conducted. The cells were incubated at 30° C. in a New Brunswick Gyrotory shaker at 150 RPM in two-liter flasks containing 1 liter of medium. Cell numbers were determined by counting in a hemacytometer after fixing in 1 volume of 10% formalin and staining with malachite green.

Table 1. Media for growth of Tetrahymena pyriformis

Medium A	Medium B	Medium C
1.5% proteose peptone	1.5% proteose peptone	0.15% proteose peptone
0.5% glucose	0.5% glucose	0.01% yeast extract
0.1% yeast extract	0.1% yeast extract	0.1% Na_2HPO_4
0.047 M NaCl	0.1% Na_2HPO_4	1/10 vol. tap water
0.001 M $MgSO_4$	1/10 vol. tap water	0.0278 M carbohydrate[a]
0.012 M Phosphate buffer, pH 7.3		

[a]Glucose in the feed portion and pentaerythritol in the starve portion of the cycle.

Cycling

Ryley (30) states that in the absence of extracellular nutrients, T. pyriformis can survive for considerable periods of time primarily at the expense of intracellular glycogen reserves. Levy and Elliott (31) point out that T. pyriformis can accumulate within a few hours large amounts of glycogen when incubated on a glucose-salts medium. The above noted observations served as the basis of several different methods for causing fluctuation in the glycogen stores of a protozoan population. Best results were obtained with the following regimen. 1. Initial growth of the population on medium B. 2. Ten hours on medium C-pentaerythritol (see Table 1).

3. Fourteen hours on medium C-glucose. Conditions 2 and 3 were repeated for as many starve-feed cycles as were desired. Cells in any particular medium were collected by centrifugation as gently and rapidly as feasible and then either resuspended in further growth or maintenance media or harvested for glycogen.

Glycogen Isolation

KOH procedure

Glycogen isolation from the cells in a small amount of culture was used to follow fluctuations in the glycogen level. The isolation was accomplished by digestion in strong alkali. The cells centrifuged from 50 ml. of culture were digested in 10 ml. of 30% KOH in a boiling water bath for 30 minutes. The digest was then cooled. Since the addition of 2 volumes of ethanol led to a biphasic system, the cooled digest was partially neutralized with acetic acid and dialized against distilled water. The dialysate was filtered, and 2 volumes of ethanol were added to the filtrate. The centrifuged glycogen was suspended in water and reprecipitated by the addition of 2 volumes of ethanol. The collected glycogen was washed with acetone and air dried.

Mercuric chloride procedure

The major method of glycogen preparation is a modification of the method of Mordoh *et al.* (7), which in turn is based upon that reported by Peat *et al.* (32) for the isolation

of phytoglycogen from sweet corn. Peat et al. stated that 0.01 N mercuric chloride was sufficient to inhibit autolytic carbohydrases. Mordoh et al. used 3 volumes of 3% mercuric chloride in the preparation of glycogen from rat livers; such concentration of mercuric chloride is 16 times that deemed adequate by Peat et al., but the solution employed by Mordoh et al. was partially neutralized to prevent acidic disruption of the glycogen α-structure. The neutralization was described as "adding as much NaOH as possible without producing a permanent yellow precipitate." As modified for use with the protozoan cells, the procedure is as follows: 1 volume of 6% mercuric chloride, adjusted to pH 4.5 with sodium hydroxide, was added with mixing to 1 volume of cell slurry. The cells were disrupted by sonication for 3 minutes using a Bronwill Biosonik at $\frac{1}{2}$ power output with optimum tuning. Debris was packed by centrifugation, resuspended in water, and collected again by centrifugation. Glycogen was precipitated by the addition of 2 volumes of ethanol to the combined supernatants. After centrifugation, the glycogen was resuspended in water. A small amount of insoluble material was removed by centrifugation, and the glycogen was reprecipitated by the addition of 2 volumes of ethanol. The collected glycogen was washed with acetone and air dried. The long sonication period indicated above was necessitated by the protective quality of the gelatinous precipitate formed from the cytoplasm of the first ruptured cells.

Gel Filtration

Broad size fractionation of glucose, oligosaccharides, and macrodextrins was achieved by use of Sephadex G-50 columns. G-50 was chosen because of its molecular weight exclusion limit of 10,000 for dextrans. Column size was 0.6 by 67 cm., and elution was with water. The applied sample contained 10 mg. total carbohydrate in 0.5 ml. Ten drop fractions were taken containing approximately 0.45 ml. Flow rate was approximately 30 sec./drop.

Analysis of effluent

The carbohydrate content of each fraction was determined by the phenol-sulfuric acid total carbohydrate method (33). After mixing the reagents manually, the absorbance was measured employing a Technicon flow-cell colorimeter with a 420 nm. interference filter. For radioactive samples, a 30λ portion was removed from each fraction and dried on a ½" square of Whatman #3MM filter paper. The dried papers were placed in toluene-PPO-POPOP counting fluid (0.4% PPO, 0.02% POPOP), and radioactivity was measured using a Packard Tri-Carb liquid scintillation spectrometer. Specific activity of each radioactive fraction was determined as cpm/30λ aliquot per absorbance of the remainder of the fraction analyzed by the phenol-sulfuric acid method.

Correlation of column elution profile and bulk alcohol precipitation

A 10 mg. sample containing at least 1% of a component at each end of the fractionation range was eluted from the above described column in 37± 2 fractions. Gel filtration of samples that had components insoluble in 66% ethanol and comparison of the elution profiles and the yields from ethanol treatment of large quantities (3 g.) of the same sample indicated that the amount of carbohydrate insoluble in 66% ethanol was the same portion of the whole as the amount of carbohydrate eluted in the first 17 column fractions following the void volume. For small samples, carbohydrate precipitable from solution by 66% ethanol was estimated by gel filtration.

Centrifugation

All centrifugal analyses were done on a Spinco Model E analytical ultracentrifuge usually using an An-D rotor and double sector synthetic boundary cell. Some experiments were conducted in the similar An-A rotor or using a single sector cell. A schlieren optical system was used. Centrifugations were done at 20° C. on aqueous solutions approximately 1% in carbohydrate. A concentration dependence of sedimentation coefficients of glycogen has been claimed (34), but other workers do not agree (35); and since the various centrifugations were done at nearly the same concentration, no correction was made for concentration dependence. Plates

were measured on a microcomparator, and sedimentation coefficients were determined from the equation

$$s = \frac{1}{60\omega^2} \cdot \frac{d\ln x}{dt}$$

where t is elapsed time in minutes, ω is angular velocity in radians/sec., x is the distance in cm. from the center of rotation to the peak, and $d\ln x/dt$ is the slope of the plot of ln x vs. t.

In instances of a bimodal sample, a representative frame was projected on graph paper, the image traced, and the relative amounts of both sized populations were calculated after planimeter measurement of the areas under the curve. The baseline was provided by the water side of the double sector cell. The demarcation between the two peaks was chosen as the line from the minimum between the peaks normal to the baseline. Corrections for radial dilution would change the relative amounts of the two components by less than 1% and, consequently, corrections for radial dilution were not made.

Graphic Determination of Molecular Weight

The magnitude of molecular weight of a glycogen or degraded glycogen population was determined graphically. Webber (36) measured the sedimentation coefficients of several mono- and oligosaccharides and empirically determined that the relation between sedimentation coefficient and molecular weight may be expressed as

$$s_{20,w} = 0.0087\ M^{0.56}.$$

Alternatively,

$$\log s_{20,w} = 0.56 \log M + \log 0.0087.$$

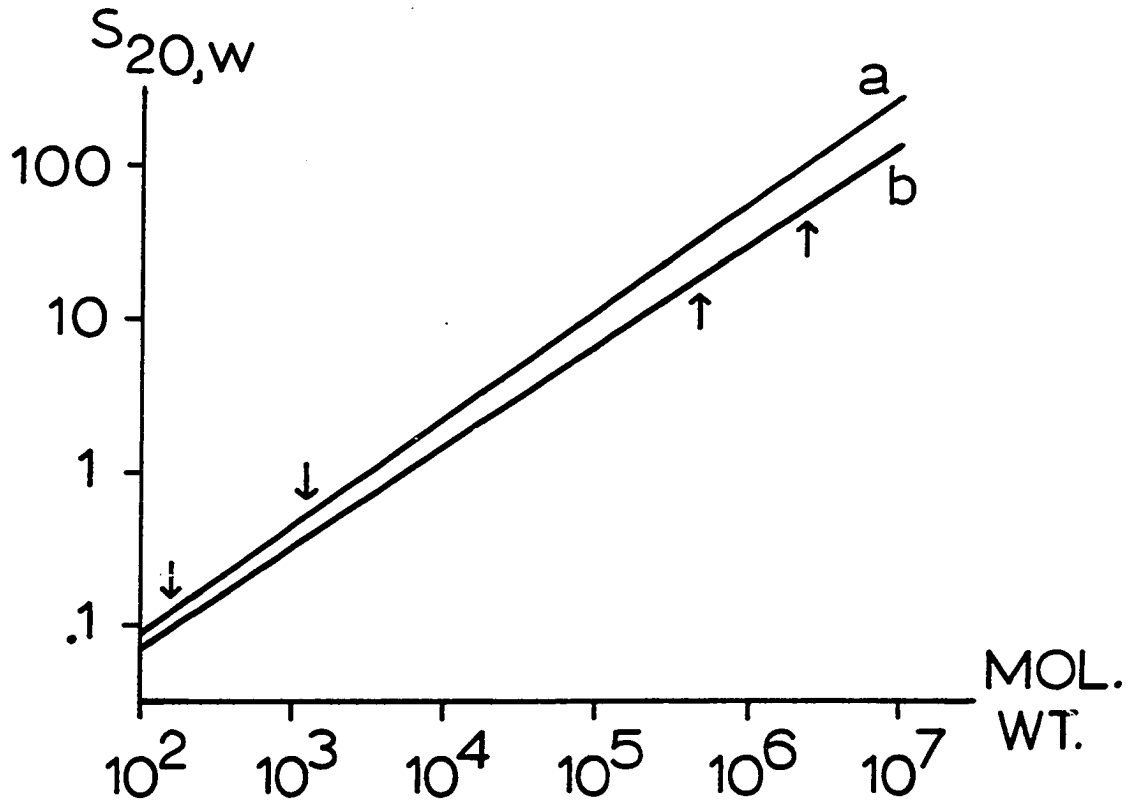
Considering only the maltosaccharides measured by Webber, the equation is

$$\log s_{20,w} = 0.70 \log M + \log 0.0034.$$

Cori (37) measured mean sedimentation and diffusion coefficients and calculated molecular weight values for a glycogen preparation and a series of phosphorylase limit dextrins. A least squares analysis of her data indicates sedimentation coefficient and molecular weight are related by the equation

$$\log s_{20,w} = 0.65 \log M + 0.0036.$$

The line based upon the Cori data and the line based upon Webber's maltosaccharide data are remarkably similar considering that the molecular weights of the materials from which the data were derived differ by a factor of approximately 10^4. A full logarithmic graph was constructed relating molecular weight and sedimentation coefficient; the lines based upon Cori's data and Webber's maltosaccharide data were plotted (Figure 1). For any observed $s_{20,w}$, the molecular weights determined by the intercepts of the two lines were taken as indicative of the range of the mean molecular weight for the observed population. A single value was calculated by taking the mean of the two values.



Line a is based upon Webber's data (36) for maltosaccharides. Line b is based upon Cori's data (37) for a glycogen and several phosphorylase limit dextrins derived from it. The arrows indicate on each line the extremes of the data points used in the construction of that line.

Figure 1. Logarithmic plot of $s_{20,w}$ vs. molecular weight

Paper Chromatography

All paper chromatography was conducted using Whatman #3MM paper, 65% aqueous propanol, ascending mode, and high temperature as described by French et al. (38).

β-Limit Dextrins

β-Limit dextrins of glycogens were prepared by buffering a 7-8% solution of substrate with sodium acetate, pH 4.65,

20 mM, containing 1 mM mercaptoacetate. β-Amylase was added, and the digest was dialized against buffer for 5 days. Solid trichloroacetic acid was added to achieve a concentration of 2%, and any sediment was removed by centrifugation. The supernatant was dialyzed against distilled water and then lyophylized.

Degradation Conditions

All α-amylolytic degradations were accomplished utilizing porcine pancreatic α-amylase. The enzyme activity was varied by factors of 10 for any serial degradation. 20 mM sodium glycerophosphate, pH 6.8, was used as a buffer. The enzyme as supplied was a crystalline suspension in half-saturated sodium chloride containing 3 mM $CaCl_2$; no additional Cl^- or Ca^{++} was used. Incubation temperature was 40° C.

Size-decrease degradation

A substrate concentration of 10 mg./ml. was used in size-decrease degradations. Three different sample sets were taken. In the first sample set, taken to follow reducing value, one ml. of sample was added to 4 ml. of 2% sodium carbonate to stop the enzyme action. Reducing values were determined by the alkaline ferricyanide procedure employing a Technicon Autoanalyzer (39). To a second sample of 2 ml. was added 4 ml. of ethanol. The precipitated material was collected and retained for centrifugal analysis. The third sample of 1 ml. was made basic with NH_4OH and frozen

for later column analysis.

Macrodextrin Production

Heller and Schramm (19) prepared macrodextrins by first hydrolyzing glycogen in a dialysis bag, then removing enzyme by trichloroacetic acid addition and centrifugation, dialyzing to remove the trichloroacetic acid, and finally reducing the final dialysate to dryness. The authors described the fractionation of their product into fractions the smallest of which was D.P. 7-50. Clearly an oligosaccharide as small as 7 glucose units is not properly described as a macrodextrin; accordingly, macrodextrin preparation was modified simply to repeated 66% ethanol precipitation of a substrate-amylase digest. Operationally, macrodextrin was therefore considered as a glycogen fragment insoluble in 66% ethanol; equivalently, macrodextrin is also that portion of carbohydrate eluted on the high molecular weight side of an empirically determined point in a Sephadex G-50 column elution profile. Paper chromatographic analysis gave no indication of compounds smaller than about 20 glucose units in a macrodextrin preparation.

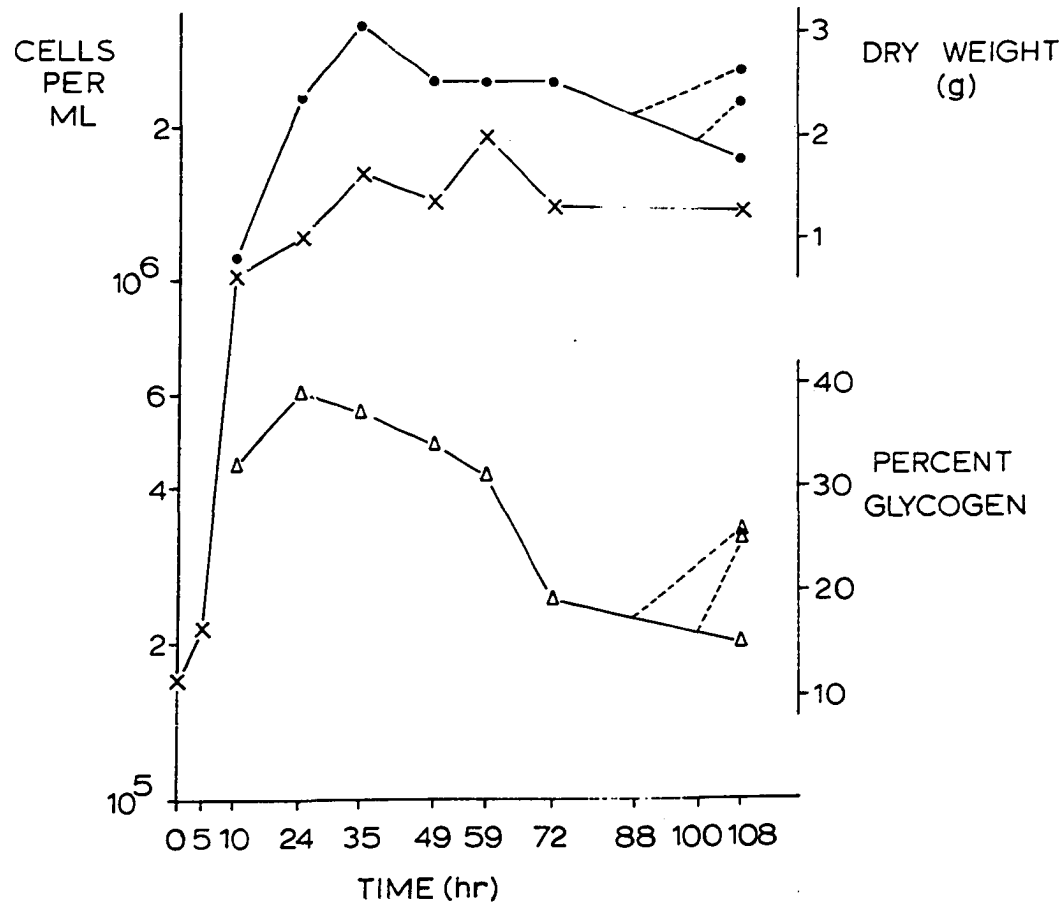
RESULTS

Growth of Organism

A set of flasks containing a culture of T. pyriformis at a density of 1.7 x 10^5 cells/ml. was incubated as described above. At various time intervals, a flask was removed, the number of cells was determined, and the cells were harvested for glycogen. Cell debris was retained and dried. Figure 2 shows the time course of cell numbers, cell dry weight per liter of culture, and dry weight percentage of glycogen. Three flasks were harvested at time 108 hours. One flask had no additions. Fifty grams of glucose were added to one flask 20 hours prior to harvest, and the same amount of glucose was added to the remaining flask 8 hours prior to harvest. The glycogen content of the cells was maximal in the early stationary phase of the culture. Accordingly, the early stationary phase was selected as the time of glycogen harvest. A representative culture of 10 l. was harvested after 42 hours at a cell density of 1.1 x 10^6 cells/ml. and yielded 8.6 g. of glycogen, 42% of the dry weight of the cells taken.

Serial Degradation

The β-limit dextrins of shellfish glycogen and protozoan glycogen were serially degraded. Figure 3 indicates a time course of the increase in reducing value for shellfish (part A) and protozoan (part B) polysaccharide. The logarithmic scale

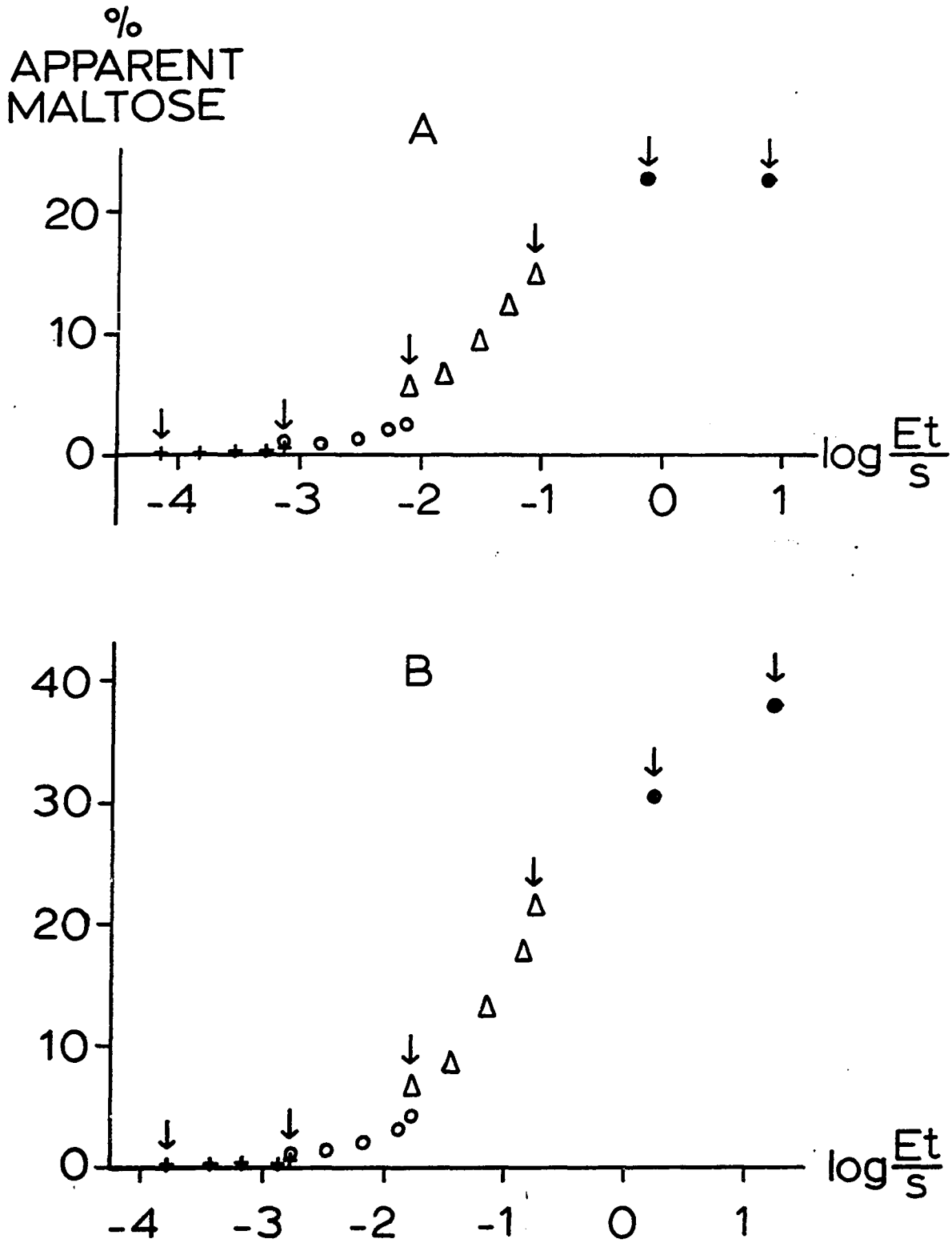


Cells per milliliter is designated by X's. The dry weight of cells in 1 liter of culture is designated by closed circles. Percent glycogen on a dry weight basis is designated by triangles. The solid line connects points representing no changes in culture conditions. The broken lines indicate changes following addition of 50 g. of glucose to 1 liter of culture either 8 or 20 hours prior to harvest.

Figure 2. Time course of T. pyriformis culture

Figure 3. Time course of glycogen β-dextrin degradation

% apparent maltose is a reducing value scale calculated by dividing 100 times the concentration of maltose with an equivalent reducing value by the concentration of original polysaccharide. Et/s is enzyme concentration in units/ml. times time in minutes divided by substrate concentration in mg./ml. The crosses, open circles, triangles, and closed circles represent samples from digests having successive decade increases in enzyme activity. The arrows indicate times at which samples were taken for column and centrifugal analysis. Part A describes shellfish polysaccharide. Part B describes protozoan polysaccharide.


%
APPARENT
MALTOSE
A
20
10
0
-4
-3
-2
-1
0
1
log Et/s
B
40
30
20
10
0
-4
-3
-2
-1
0
1
log Et/s

allows a singular presentation of differences in polysaccharide exposure to enzyme varying by factors of 10^5 or more. The enzyme activity in International units was determined in each case using as substrate the polysaccharide being studied. (One unit is that amount of enzyme which will catalyze the transformation of one micro-equivalent of the group concerned per minute under defined experimental conditions.) Equal amounts of enzyme protein using protozoan polysaccharide as substrate had an activity 2.3 times the activity using shellfish polysaccharide as substrate.

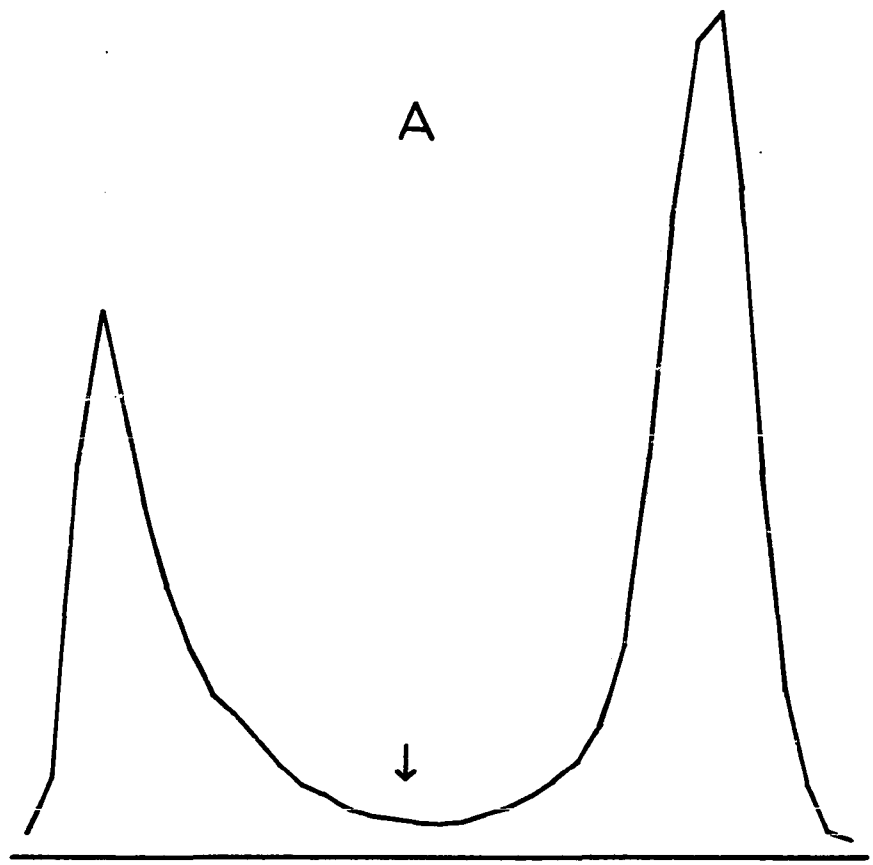
Column and Centrifugal Analyses

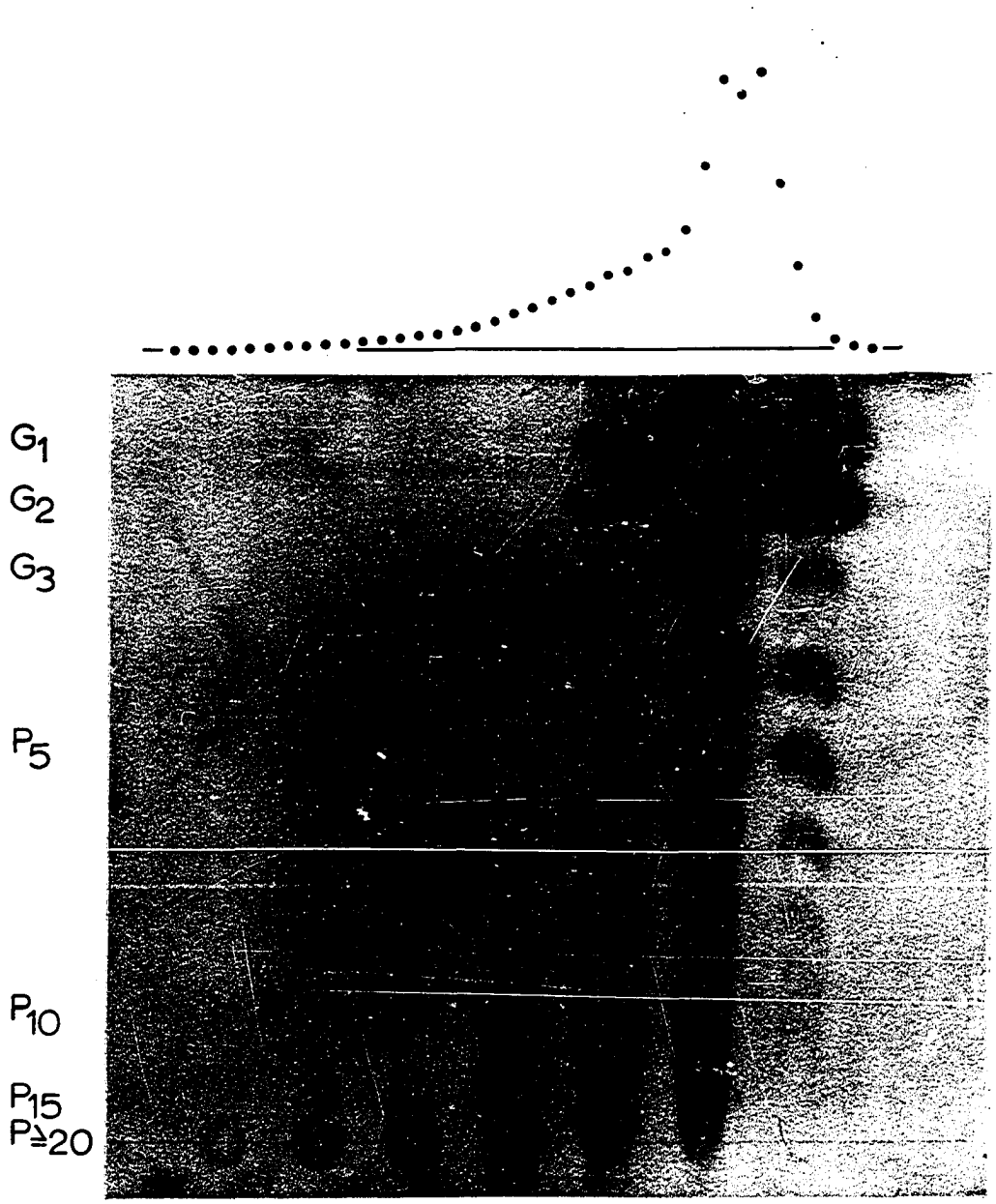
Figures 4 and 5 portray representative results obtained employing Sephadex G-50 gel filtration. Figure 6 shows some representative schlieren patterns from ultracentrifugation.

Table 2 compiles the various parameters measured on the shellfish glycogen β-dextrin and its degraded forms, and Table 3 presents similar measurements on the protozoan glycogen β-dextrin and its degraded forms. In other experiments utilizing shellfish and protozoan glycogens rather than their β-dextrins, the percentages of the wholes represented by the macrodextrins, as measured by column analysis, were as low as 5.5 and 0.5 respectively for shellfish and protozoan polysaccharide. Since the β-dextrin represents about $\frac{1}{2}$ of the original glycogen, values for the amount of eventually produced macrodextrin on a scale comparable to that of Tables 2 and 3

Figure 4. Representative elution profiles from Sephadex G-50 columns

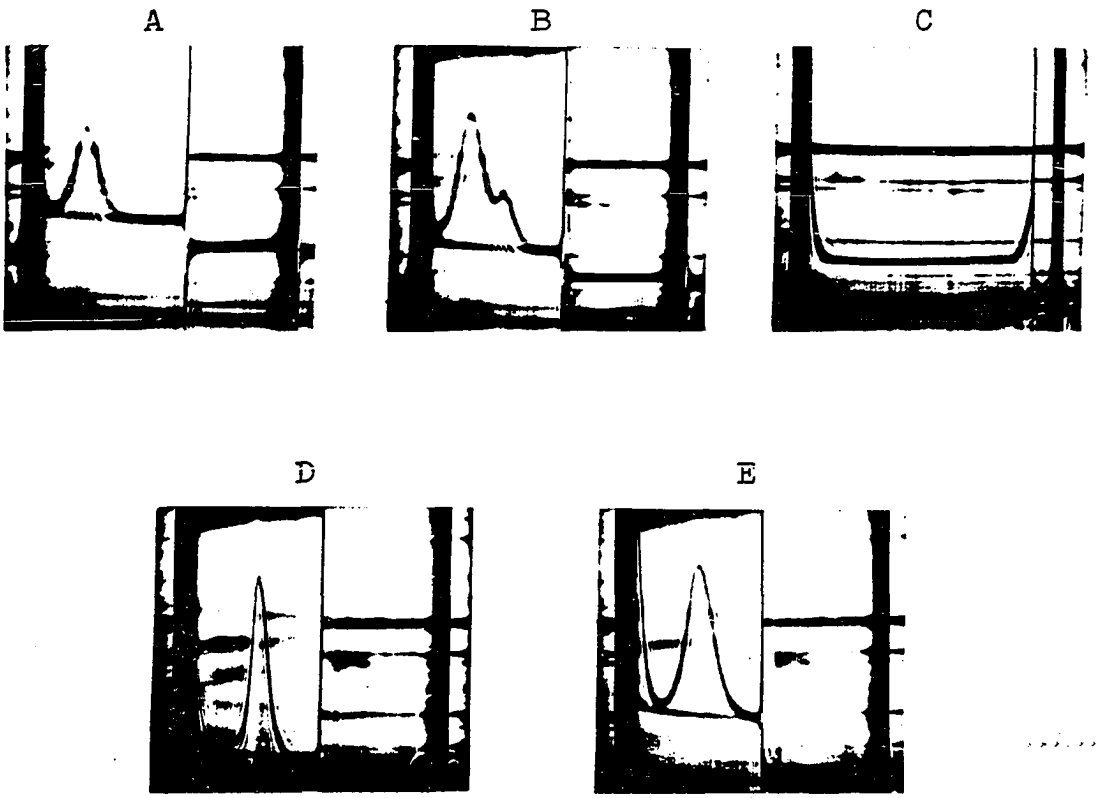
Both profiles are to the same arbitrary scales. Ordinate is carbohydrate content. Abscissa is elution volume. Molecular weight of the eluted material decreases from left to right. The arrow indicates empirically determined 66% ethanol insoluble-soluble demarcation point. Part A is the elution profile of shellfish glycogen partially degraded by Aspergillus oryzae α-amylase. Part B is the elution profile of the 66% ethanol insoluble portion of the same degraded glycogen. 97% of the applied carbohydrate was eluted on the high molecular weight side of the demarcation point.


A
B



Every fifth fraction from a G-50 column separation of an amylase-degraded shellfish glycogen was spotted on this chromatogram. G_1, G_2, and G_3 designate respectively glucose, maltose, and maltotriose. P_x designates an oligosaccharide of x glucose units. The elution profile above the chromatogram is scaled to coincide with the chromatogram. Correlation between position in elution profile and range of oligosaccharides up to about D. P. 15 at that point can be seen.

Figure 5. Chromatogram of selected column fractions



All samples are original or variously degraded protozoan glycogen β-dextrin. See Table 3 for a numerical description of the samples whose sedimentation appearance is shown in this figure. Direction of sedimentation is from right to left. Parts A, B, D, and E show samples in double sector synthetic boundary cells; part C is a single sector cell. Part A shows the undegraded polysaccharide after 7 minutes at 20,000 RPM; there is no evidence of a low molecular weight fraction in the original β-dextrin. Part B shows the least degraded sample after 2.5 minutes at 30,000 RPM. This frame is the one from which the relative amounts of the large and small populations in this sample were determined. Part C shows the sample taken at log Et/s = -0.77 after 9 minutes at 30,000 RPM. There is no indication of a high molecular weight population at this stage of degradation. The small population peak has not moved away from the meniscus and would not do so under these conditions. Part D shows the most degraded sample after 1 minute at 52,000 RPM, and part E shows the same sample 12 minutes later.

Figure 6. Representative schlieren patterns

Table 2. Shellfish glycogen β-dextrin and its degraded forms

log Et/s	% Apparent Maltose	% Soluble in 66% Ethanol	$s_{20,w}$ Large Comp.[a]	M.W.[b] x 10-6	% of 66% Ethanol Insol.	% of Total (Lg.)	$s_{20,w}$ Small Comp.	M.W.	% of 66% Ethanol Insol.	% of Total (Sm.)
Undegraded	---	0	51	1.6	100	100	N.P.[c]	---	0	0
-4.23	0.17	7	44	1.24	82.4	76.5	N.D.[d]	---	17.6	16.4
-3.23	0.17	11	42	1.1	71.6	63.7	1.6	9300	28.4	25.2
-2.23	5.4	23	31	0.8	12.4	9.5	N.D.	---	87.6	67.5
-1.23	14.6	48	20[e]	---	3	1.6	N.D.	---	97	50.5
-0.23	22.5	72	N.P.	---	0	0	1.4	7600	100	28
0.87	22.5	74	N.P.	---	0	0	N.D.	---	100	26

[a]Comp. ≡ component.

[b]Determined graphically.

[c]Not present.

[d]Not determined.

[e]Estimate.

Table 3. T. pyriformis glycogen β-dextrin and its degraded forms

log Et/s	% Apparent Maltose	% Soluble in 66% Ethanol	$s_{20,w}$ Large Comp.[a]	M.W.[b] x 10^{-6}	% of 66% Ethanol Insol.	% of Total (Lg.)	$s_{20,w}$ Small Comp.	M.W. x 10^{-3}	% of 66% Ethanol Insol.	% of Total (Sm.)
Undegraded	---	0	116	7.2	100	100	N.P.[c]	---	0	0
-3.77	0.13	12.5	102	4.4	77	67	2.0	13	23	20
-2.77	0.95	19.2	53	1.6	10	8	1.3	7	90	73
-1.77	6.7	T H I S	S A M P L E			W A S		L O S T		
-.77	21.3	64	N.P.	---	0	0	N.D.[d]	---	100	36
0.23	30.4	83	N.P.	---	0	0	N.D.	---	100	17
1.23	37.9	88	N.P.	---	0	0	0.7	3	100	12

[a]Comp. ≡ Component.

[b]Determined graphically.

[c]Not present.

[d]Not determined.

would be about 11% as a lowest observed value for the column headed "% of Total (Sm.)" in Table 2 and about 1% as a lowest observed value for the same column in Table 3.

Figures 7 and 8 present in an easily visualized fashion the fragmentation pattern of shellfish glycogen β-dextrin and protozoan glycogen β-dextrin. The early production of a small component from both polysaccharides and the proportion of the whole it represents can be seen; the rapid decrease in size and amount of the parent polysachharide can also be observed.

Cycling

Figure 9 describes the fluctuation of the glycogen level in a population of T. pyriformis. Cell numbers remained constant throughout the cycling process. About 40% of the final glycogen was synthesized from ^{14}C labeled glucose. β-amylase was able to release as maltose only about 1/3 of the label.

Degradation and Analysis of Cycled Glycogen

The β-dextrin of the glycogen whose cycling history is depicted in Figure 9 was degraded serially, using decade increases in exposure of polysaccharide to enzyme such that the log Et/s for the most extensive degradation was approximately 1. Each mixture was fractionated by gel filtration, and the specific activity of each column fraction was determined. Figure 10 indicates the specific activity vs.

fraction number for each digest. The β-dextrin was chosen for degradation since interest was in branched regions, and the large amount of polymer removable by β-amylase tends to mask the observation of the specific activity of singly-branched regions. Of particular interest is part D of Figure 10; this part shows that the specific activity decreases and eventually becomes indistinguishable from zero with increasing size.

Figure 7. Three-dimensional graph of the fragmentation pattern of shellfish glycogen β-dextrin

The $s_{20,w}$ axis is straightforward. The solubility values were determined by gel filtration analysis. Amount (%) is the proportion of the total carbohydrate represented by each component. Populations of two different size ranges are represented. The crosses are on the $s_{20,w}$-solubility plane. The solid line between crosses describes the course of size change with solubility. With the exception of the point representing the undegraded β-dextrin, all circles represent points in 3 dimensions. The broken lines add the dimension of amount to components of any given size. The solid lines connecting the circles describe the change in the amount and size with the solubility reaction parameter of either the parent polysaccharide or the macrodextrin.

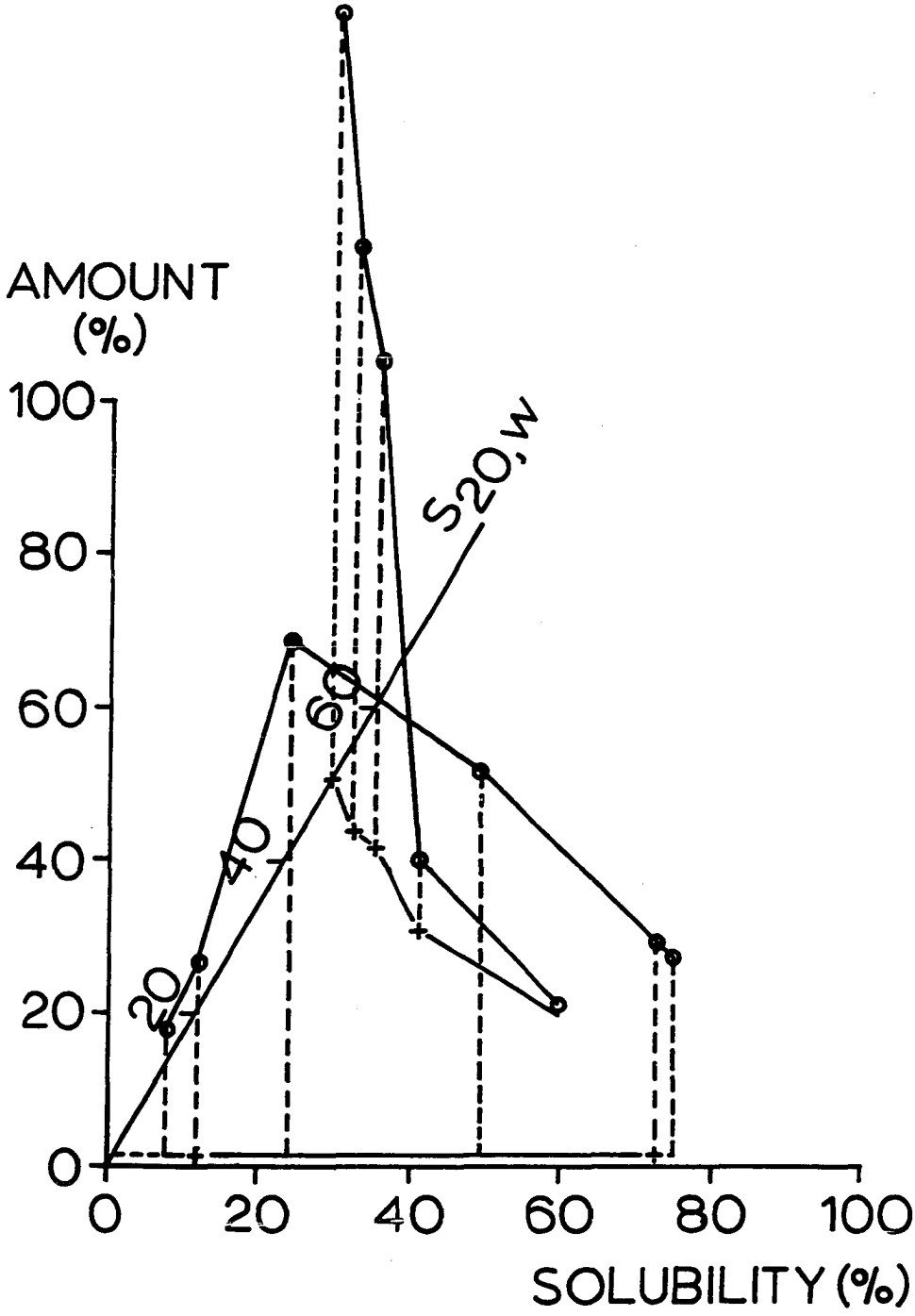
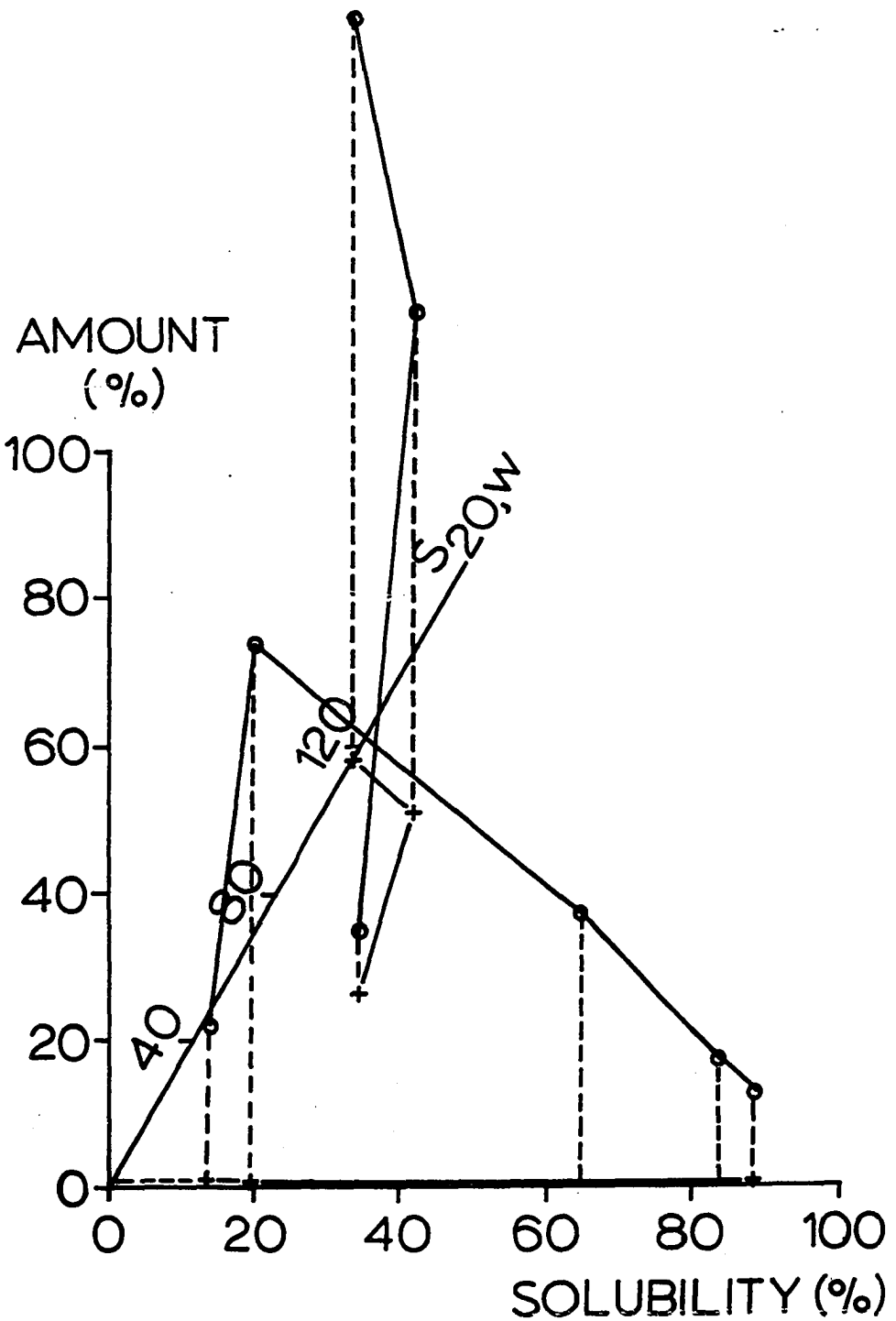
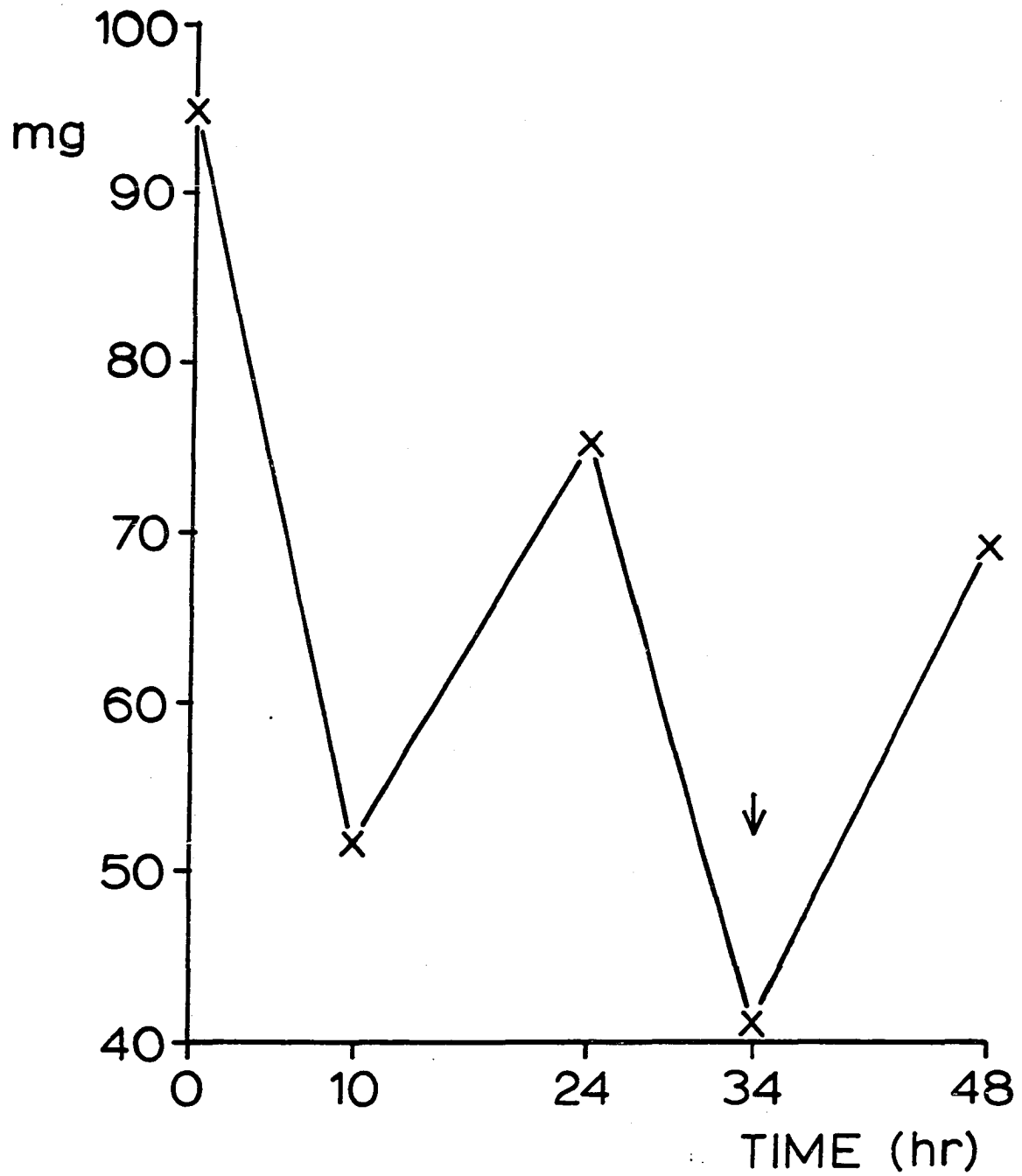

AMOUNT
(%)
100
80
60
40
20
0
0
20
40
60
80
100
SOLUBILITY (%)
$S_{20,w}$
20
40
60

Figure 8. Three-dimensional graph of the fragmentation pattern of protozoan glycogen β-dextrin

The $s_{20,w}$ axis is straightforward. The solubility values were determined by gel filtration analysis. Amount (%) is the proportion of the total carbohydrate represented by each component. Populations of two different size ranges are represented. The crosses are on the $s_{20,w}$-solubility plane. The solid line between crosses describes the course of size change with solubility. With the exception of the point representing the undegraded β-dextrin, all circles represent points in 3 dimensions. The broken lines add the dimension of amount to components of any given size. The solid lines connecting the circles describe the change in the amount and size with the solubility reaction parameter of either the parent polysaccharide or the macrodextrin.


AMOUNT
(%)
100
80
60
40
20
0
0
20
40
60
80
100
SOLUBILITY (%)
S20W
120
60
40

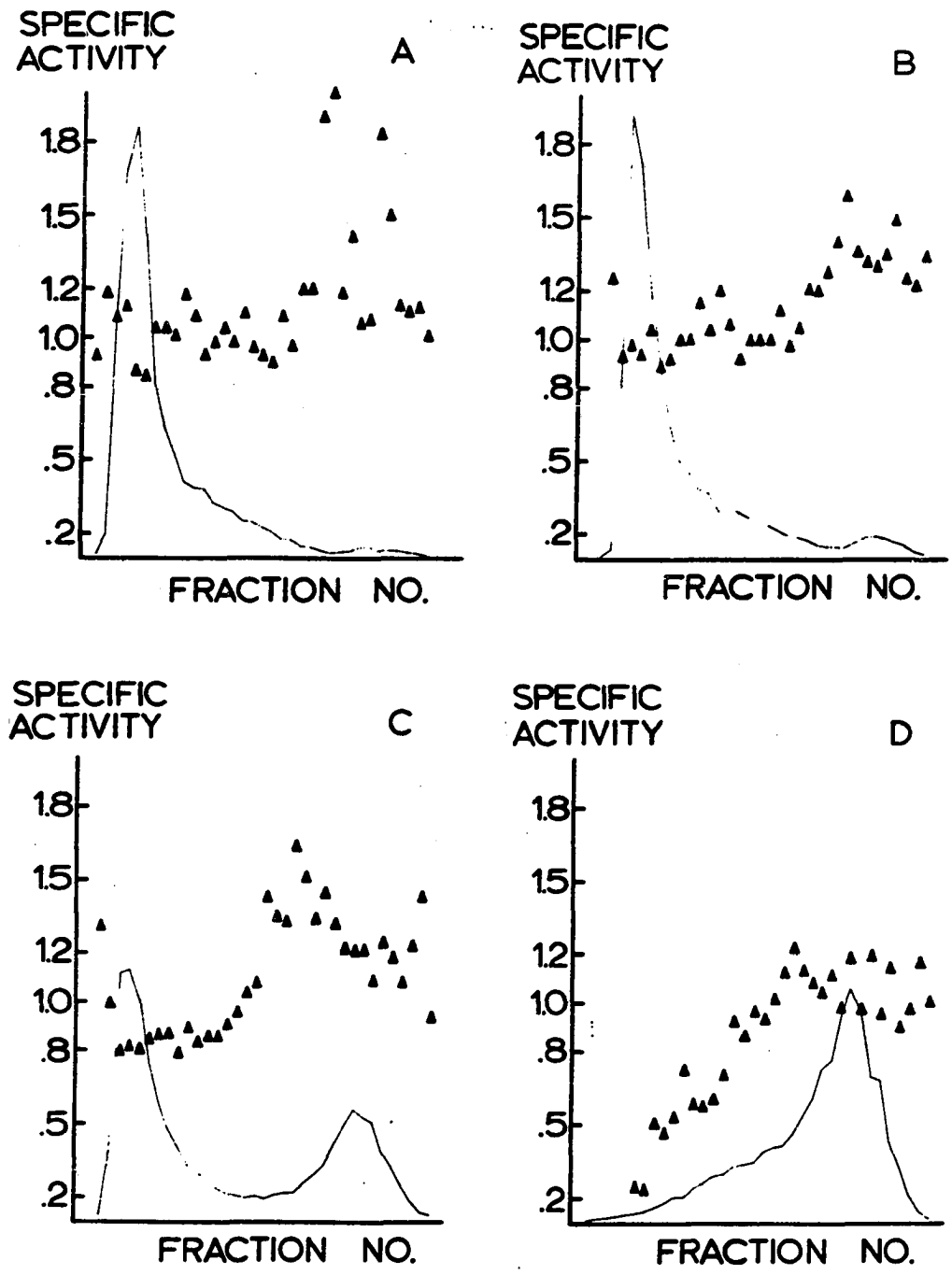


The time scale starts at the beginning of the first fasting period; the culture was 35 hours old at that time. The glycogen content is that amount of glycogen harvested from the cells in 50 ml. of culture. The arrow indicates the point of addition of ^{14}C labeled glucose for the final feeding period.

Figure 9. Glycogen level fluctuation.

Figure 10. Specific activity of each Sephadex G-50 column fraction from the separation of differently degraded portions of protozoan glycogen β-dextrin

Parts A, B, C, and D differ by decade increases of exposure of polysaccharide to enzyme such that the log Et/s for part D was approximately 1. The normalized specific activity of each column fraction is indicated by a triangle. Superimposed on each plot is the carbohydrate elution profile for that sample. Specific activity, initially determined as cpm/30λ portion of the fraction per absorbance of the remainder of the fraction as analyzed by the phenol-sulfuric acid method, was normalized to the specific activity of the whole fractionated sample as determined as the sum of the cpm of each 30λ portion per the sum of the absorbances of all the fractions. All indicated specific activities were calculated from radioactivity values greater than background with a probability >99% and absorbance values greater than the absorbance produced by 5γ glucose (equivalent to 0.05% of the total sample).


SPECIFIC ACTIVITY
A
1.8
1.5
1.2
1.0
.8
.5
.2
FRACTION NO.
SPECIFIC ACTIVITY
B
1.8
1.5
1.2
1.0
.8
.5
.2
FRACTION NO.
SPECIFIC ACTIVITY
C
1.8
1.5
1.2
1.0
.8
.5
.2
FRACTION NO.
SPECIFIC ACTIVITY
D
1.8
1.5
1.2
1.0
.8
.5
.2
FRACTION NO.

DISCUSSION AND CONCLUSIONS

One object of these studies was to answer the question of where in the glycogen molecule macrodextrin-forming regions occur. The alternatives seemed to be two. The placement of macrodextrin regions in glycogen could be described in some statistical fashion; the densely-branched regions would be separated by less densely branched, more easily hydrolyzed regions. The second alternative calls for an increasing branching density from the periphery to the center of the molecule. Consider a model for synthesis such that the molecule after the first branch point is formed may be approximated by a sphere of radius r. Each chain end would then have an area of domain on the surface of the sphere of $2\pi r^2$. Assume that a domain of, say, πr^2 is the minimum necessary to allow for placement of a branch on that chain and, further, that the number of chain ends increases approximately as the powers of 2. A second set of branches, resulting in 4 chain ends, would require no further elongation of the chains receiving the branches. The third set of branches would require prior chain elongation of 0.414 r in order to attain the minimum surface area domain. Successive additional sets of branches would require progressively greater increases in the radius of the approximating sphere. In this fashion, the branches near the center of the molecule would be closer together than those nearer the periphery.

Glycogen synthesized according to such a model would possess macrodextrin-like regions at the center of the molecule.

Macrodextrins are products of α-amylase action upon glycogen. β-dextrins contain all the branch points of the parent glycogen; and, since interest is in regions around branch points, the branch-enriched β-dextrin was selected for quantitative study. Preliminary experiments on whole shellfish glycogen had qualitatively indicated rapid fragmentation by α-amylase, and the β-dextrin was used simply to enrich the amount of branching in the mixtures being examined.

Figure 7 presented three-dimensionally the course of rapid fragmentation of the parent β-dextrin of shellfish glycogen and the early appearance of small particles. The small particle is not a macrodextrin in that it is not a limit dextrin. More extensive degradation further decreases the amount and size of the small particle. The small component is a precursor of whatever macrodextrin is eventually produced in that it contains the amylase-resistant branching arrangements of which the macrodextrin is composed.

The shellfish glycogen used in these experiments is a commercial product prepared apparently by extraction of mussel tissue with hot, concentrated alkali; such drastic procedures are known to cause at least gross physical degradation. There is also some indication that alkali treatment changes the behavior of the glycogen as an enzyme

substrate. There seems to be nothing reported regarding possible fine structure changes resulting from alkali treatment. In addition, nothing is known of the metabolic state of the tissue which was extracted nor was anything known of the history of the glycogen. Since the physical and biological properties of a glycogen depend upon tissue of origin, method of extraction, and metabolic state of the tissue (12), examination of at least one other glycogen was thought necessary. In addition to using a different tissue of origin, a milder method of extraction was used. A source in which the metabolic state could be controlled or at least in which the state would be known was desired. A source in which the metabolic history could also be controlled was desired in the hope that some light would be shed on the origin of macrodextrins. A protozoan source was thought to fulfill most appropriately the above criteria, and Tetrahymena pyriformis was selected for study. A short life cycle, the large number of individuals, and single-celledness are all features of advantage.

The glycogen harvested from T. pyriformis cells in the early stationary phase is newly synthesized. The three-dimensional presentation of the fragmentation pattern of protozoan glycogen β-dextrin, Figure 8, shows the fragmentation of such newly synthesized glycogen. Presumably, this glycogen has not been exposed to the fluctuations of levels of enzyme activities and of nutritional state, such as the

glycogen in a mammalian liver might be exposed to, which might alter the initial branching pattern. Figure 8 shows the rapid fragmentation of the parent β-dextrin and the early appearance of small, macrodextrin-precursor particles. On the basis of the results indicated in Figure 7 and Figure 8, it is concluded that macrodextrins arise from randomly arranged densely-branched regions in shellfish glycogen and T. pyriformis glycogen. A correlate of this conclusion is that there is more than one macrodextrin-forming region per glycogen molecule. Consider the size and yield data for the small components from Table 2 and Table 3. The molecular weight of the small component from shellfish polysaccharide may be estimated at 7500 when it accounts for 26% of the original material. From the tabular values and the estimate, it may be calculated that there are about 55 densely-branched, small component precursor regions per original shellfish β-dextrin molecule of 1.6×10^6 molecular weight. Similarly, there are about 300 densely-branched, small component precursor regions per original T. pyriformis β-dextrin molecule of 7.2×10^6 molecular weight.

It would seem reasonable to expect a similar arrangement of branches in many other glycogens. The similarly constituted amylopectin could also possess a random arrangement of branching density. Some glycogens might be expected to have a different arrangement of branching density. One such

glycogen might be the glycogen found in persons suffering from that glycogenosis in which glycogen synthetase seems to be absent (40). In the case of glycogen synthetase deficiency, whether there is total lack of the enzyme or only a small amount of it present, the relative branching activity would be very much higher than that obtaining under "normal" conditions of synthesis. With such a high relative branching activity, steric factors could be the limiting determinant of the distribution of branching, and the resulting glycogen could have a high central branching density with decreasing branching density towards the periphery of the molecule. In this regard, it would be informative to examine glycogens synthesized in vitro with a gradation of branching enzyme relative to elongating enzyme such as those described by Smith (29). Another glycogen possibly not possessing a random pattern of branching density is that glycogen produced by Aerobacter aerogenes A3(S1) and described by Kindt and Conrad (41). These authors report that the β-limit dextrin derived from the A3(S1) glycogen has an internal chain length of 0.9. Such a β-dextrin could in itself be one super macrodextrin.

The second object of these studies was to define some factors which affect the amount and placement of those densely-branched regions which result in macrodextrins. Variation in the amount of macrodextrin produced from different tissues cannot be claimed if the history of the

tissue is not known. Examination of Figures 7 and 8 indicates that the parent particle of shellfish glycogen is more resistant to amylolysis than is the parent particle of protozoan glycogen. But the history of the shellfish glycogen is unknown. It could be that the fragmentation pattern of newly synthesized shellfish glycogen would be indistinguishable from that of newly synthesized T. pyriformis glycogen. Examination of various glycogens without a knowledge of their metabolic history would seem to be fruitless.

One possible explanation of the origin of macrodextrins could be different relative activities of branching and synthetase enzymes. It is clear from the results of Smith (29) that, up to a point, the amount of branching in a synthetic glycogen is dependent upon the relative activities of branching and synthetase enzymes; but such considerations could apply only to a newly synthesized glycogen. Glycogen in vivo, in addition to synthetase and branching enzymes, is exposed to phosphorylase, debranching activity, and amylase at least. It is conceivable that dense branching is a result of random placement of branches within the specificity limits of the branching enzyme. A minor variation of this concept adds that the amount of dense branching produced during any one synthetic cycle is small but that several periods of glycogenesis would result in a glycogen with a relatively large amount of dense branching. The more

amylase-resistant shellfish glycogen compared with T. pyriformis glycogen (compare Figures 7 and 8) could result simply from the shellfish glycogen being the product of several net synthesis periods between which net degradation periods occur. If, once formed, a complex branching array has a resistance to in vivo degradative forces similar to its resistance to amylolysis as described in this work, then the amount of such arrays would increase with time. Such a possibility is implicit in the results and hypothesis of Huijing et al. (28) regarding amylopectinosis. The accrual of dense branching would be independent of whether the branching and debranching enzymes acted cyclically or in concert. Figure 9 portrayed the cycling of the glycogen stores in a population of T. pyriformis. The cycling was conducted for the purpose of exposing the initial glycogen stores to branching and debranching enzymes for a period that was long in comparison to the time neccessary for the synthesis of those stores. Alternation of lean and rich media was employed to induce fluctuation simulating the natural fluctuation of the glycogen stores as in, for example, a mammalian liver. Also some way of distinguishing between glycogen initially present and that synthesized late in the cycling period was desired; a discrete glycogenesis period in the presence of ^{14}C labeled glucose would accomplish this aim. In early experiments, glycogen had been harvested at various time intervals after optimum glycogen deposition

(several of the samples taken at times indicated in Figure 2 were examined). The glycogen had been exposed to equivalent amounts of enzyme protein, and the degraded glycogen had been examined by gel filtration. The change in branching complexity, as measured by different relative amounts of carbohydrate on the high molecular weight side of an empirical point in the column elution profile, was less than the column reproducibility factor of 5.4%. The more sensitive assay of determining the specific activity of material handled identically (the mixture of old glycogen and newly synthesized ^{14}C labeled glycogen resulting from the cycling process) was chosen. The result clearly indicated in Figure 10 is that the extent of complex branching arrays in the newly synthesized glycogen is less than the amount of complex branching arrays in older glycogen. Part C of Figure 10 indicates a 40% higher specific activity for column fractions which Figure 5 indicates are composed primarily of singly-branched oligosaccharides. Part D of Figure 10 indicates a specific activity indistinguishable from zero for column fractions containing saccharides not mobile under conditions of paper chromatography (see Figure 5). On the basis of the claim by Pharmacia of a molecular weight exclusion limit of 10,000 for Sephadex G-50, the saccharides containing no ^{14}C label cannot be much larger than D.P. 60.

Unfortunately, the result is not definitive. The result could be explained as a different relative activity of

branching and synthetase enzymes at the time of synthesis of the ^{14}C labeled glycogen as distinguished from the relative activities at the time of the synthesis of the initial glycogen stores. If the relative activity of branching and synthetase enzymes are very nearly the same at the different times or if the relative activity does not bear upon the formation of complex, macrodextrin-like branching arrays, then the result indicated in Figure 10 would have to be interpreted as the ability of branching enzyme to form arrangements of branching which debranching enzyme cannot degrade.

It seems to this author that further experimentation with T. pyriformis could be very productive. Enzyme activites could be measured under different culture conditions and different treatments by methods described by Cook et al. (42). The methodology of Smith (29) could be applied to enzymes obtained from the protozoan source, and glycogen then synthesized in vitro. The role that relative activity of branching and synthetase enzymes have in the production of complex branching arrays could be established. The specificity of the branching enzyme in terms of where a new branch is formed in relation to pre-existing branches and the ascertainment of whether branching and debranching enzymes are subject to control would be long-range goals.

SUMMARY

On the basis of early fragmentation during α-amylolysis, it is concluded that a commercially available shellfish glycogen and a newly synthesized Tetrahymena pyriformis glycogen both possess a random distribution of α-1,6 branching bonds. It would seem reasonable to expect most other glycogens to possess a similar, random branching arrangement, but some particular glycogens which might be expected not to possess random branching are the glycogen occurring in glycogen synthetase deficiency disease and the glycogen from Aerobacter aerogenes A3(S1).

After metabolically ageing the glycogen stores of a population of T. pyriformis by alternate feeding and fasting, a period of glycogen synthesis was induced in the presence of ^{14}C labeled glucose. The mixture of old glycogen and newly synthesized, ^{14}C labeled glycogen was degraded to different extents by α-amylase, and each partially degraded glycogen was fractionated by gel filtration. Specific activity measurements of each column fraction and comparison of specific activity and total carbohydrate elution profiles for each partially degraded glycogen suggest that newly synthesized glycogen is less complexly-branched than glycogen which has had more exposure to branching and debranching enzymes.

LITERATURE CITED

1. Manners, D. J. Molecular structure of glycogens. Advances in Carbohydrate Chem. 12: 261. 1957.

2. Whelan, W. J. and Cameron, Margaret P., eds. Control of glycogen metabolism. London, J. & A. Churchill, Ltd. 1964.

3. Whelan, W. J., ed. Control of glycogen metabolism. New York, Academic Press, Inc. 1968.

4. Orrell, S. A., Jr. and Bueding, E. Sedimentation characteristics of glycogen. J. Am. Chem. Soc. 80: 3800. 1958.

5. Lazarow, A. Particulate glycogen: a submicroscopic component of the guinea pig liver cell. Anat. Record 84: 31. 1942.

6. Drochmans, P. Techniques for the isolation of particulate glycogen and its examination in the electron microscope. Biochem. Soc. Symposia 23: 127. 1963.

7. Mordoh, Jose, Krisman, Clara R., and Leloir, Luis F. Further studies on high molecular weight liver glycogen. Arch. Biochem. Biophys. 113: 265. 1966.

8. Bueding, Ernest and Orrell, Stanley A. Mild procedure for the isolation of polydisperse glycogen from animal tissues. J. Biol. Chem. 239: 4018. 1964.

9. Orrell, Stanley A. and Bueding, Ernest. Comparison of products obtained by various procedures used for the extraction of glycogen. J. Biol. Chem. 239: 4021. 1964.

10. Drochmans, P. Morphologie du glycogène. J. Ultrastruct. Res. 6: 141. 1962.

11. Stadhouders, A. M. Particulate glycogen. Nijmegen, Thoben Offset. 1965.

12. Childress, Charles C., Sacktor, Bertram, Grossman, I. William, and Bueding, Ernest. Isolation, ultrastructure, and biochemical characterization of glycogen in insect flight muscle. J. Cell Biol. 45: 83. 1970.

13. French, Dexter. Physical and chemical structure of starch and glycogen. In Schultz, H. W., Cain, R. F., and Wrolstad, R. W., eds. Symposium on foods: Carbohydrates and their roles. Pp. 26-54. Westport, Conn., AVI Publishing Co., Inc. 1969.

14. Meyer, Kurt H. Chemistry of glycogens. Advances in Enzymol. 3: 109. 1943.

15. Bailey, J. M. and Whelan, W. J. Physical properties of starch. 1. Relationship between iodine stain and chain length. J. Biol. Chem. 236: 969. 1961.

16. Robyt, John and French, Dexter. Action pattern and specificity of an amylase from Bacillus subtilis. Arch. Biochem. Biophys. 100: 451. 1963.

17. Hughes, R. C., Smith, E. E., and Whelan, W. J. Structure of a pentasaccharide α-limit dextrin formed from amylopectin by Bacillus subtilis α-amylase. Biochem. J. 88: 63p. 1963.

18. Roberts, P. J. P. and Whelan, W. J. Mechanism of carbohydrase action. 5. Action of human salivary α-amylase on amylopectin and glycogen. Biochem. J. 76: 246. 1960.

19. Heller, J. and Schramm, M. α-Amylase limit dextrins of high molecular weight obtained from glycogen. Biochim. et Biophys. Acta 81: 96. 1964.

20. Rozenfel'd, E. L. Polyglucosides resulting from action of liver glycogen α-amylolysis. Biochemistry (USSR) 23: 832. 1958.

21. Bridgman, Wilbur B. Some physical chemical characteristics of glycogen. J. Am. Chem. Soc. 64: 2349. 1942.

22. Orrell, S. A., Jr., Bueding, E., and Reissig, Magdelena. Physical characteristics of undegraded glycogen. In Whelan, W. J. and Cameron, Margaret P., eds. Control of glycogen metabolism. Pp. 29-52. London, J. & A. Churchill, Ltd. 1964.

23. Reissig, Magdelena and Colucci, Anthony V. Localization of glycogen in the cestode, Hymenolepis diminuta. J. Cell Biol. 39: 754. 1968.

24. Antione, Alan D. and Tepper, Byron S. Characterization of glycogens from mycobacteria. Arch. Biochem. Biophys. 134: 207. 1969.

25. Barber, Albert A., Harris, Warren W., and Anderson, Norman G. Isolation of native glycogen by combined rate-zonal and isopycnic centrifugation. Nat. Cancer Inst. Monograph 21: 285. 1965.

26. Meyer, Kurt H., ed. Natural and synthetic high polymers. 2nd ed. New York, Interscience Publishers, Inc. 1950.

27. Discussion on diagram of glycogen synthesis and degradation. In Whelan, W. J. and Cameron, Margaret P., eds. Control of glycogen metabolism. Pp. 401-412. London, J. & A. Churchill, Ltd. 1964.

28. Huijing, F., Lee, E. Y. C., Carter, J. H., and Whelan, W. J. Branching action of amylo-1,6-glucosidase/oligo-1,4→1,4-glucantransferase. Fed. Europ. Biochem. Soc. Letters 7: 251. 1970.

29. Smith, E. E. Enzymic control of glycogen structure. In Whelan, W. J., ed. Control of glycogen metabolism. Pp. 203-213. New York, Academic Press, Inc. 1968.

30. Ryley, J. F. Studies on the metabolism of the protozoa. 3. Metabolism of the ciliate Tetrahymena pyriformis (Glaucoma pyriformis). Biochem. J. 52: 483. 1952.

31. Levy, Michael R. and Elliott, Alfred M. Biochemical and ultrastructural changes in Tetrahymena pyriformis during starvation. J. Protozool. 15: 208. 1968.

32. Peat, Stanley, Whelan, W. J., and Turvey, J. R. Soluble polyglucose of sweet corn (Zea mays). J. Chem. Soc. 1956: 2317. 1956.

33. Dubois, Michel, Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, Fred. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28: 350. 1956.

34. Larner, J., Ray, B. Roger, and Crandall, H. F. Pattern of action of crystalline muscle phosphorylase on glycogen as determined from molecular size distribution studies. J. Am. Chem. Soc. 78: 5890. 1956.

35. Bryce, W. A. J., Cowie, J. M. G., and Greenwood, C. T. Sedimentation behavior of the components of potatoe starch in dilute alkali. J. Polymer Sci. 25: 251. 1957.

36. Webber, Russell V. Sedimentation coefficient determinations with some sugars and dextrins. J. Am. Chem. Soc. 78: 536. 1956.

37. Cori, Gerty T. Enzymatic structure analysis and molecular weight determinations of polysaccharides. Makromol. Chem. 20: 169. 1956.

38. French, Dexter, Mancusi, Joseph L., Abdullah, Mukhtar, and Brammer, Gary L. Separation of starch oligosaccharides by high temperature paper chromatography. J. Chromatography 19: 445. 1965.

39. Hoffman, W. S. Rapid photoelectric method for the determination of glucose in blood and urine. J. Biol. Chem. 120: 51. 1937. As modified in Micro-glucose. Technicon Autoanalyzer Methodology N-9. Chauncey, New York, Technicon Instruments Corp. 1964.

40. Spencer-Peet, J., Lewis, G. M., and Stewart, K. M. Glycogen synthetase deficiency. In Whelan, W. J. and Cameron, Margaret P., eds. Control of glycogen metabolism. Pp. 377-386. London, J. & A. Churchill, Ltd. 1964.

41. Kindt, T. J. and Conrad, H. E. Role of primer in glycogen biosynthesis in Aerobacter aerogenes. Biochem. 6: 3718. 1967.

42. Cook, David E., Rangaraj, Narayan I., Best, Norman, and Wilken, David R. Conversion of glucose to glycogen in Tetrahymena pyriformis. Arch. Biochem. Biophys. 127: 72. 1968.

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